Influence of Epidemiological Factors on the Bioherbicidal Efficacy of *Phomopsis amaranthicola* on *Amaranthus hybridus*

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ABSTRACT

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Greenhouse experiments were performed to determine the effect of dew period temperature and duration, plant growth stage, conidial concentration, and the addition of adjuvants on the bioherbicidal efficacy of *Phomopsis amaranthicola* on *Amaranthus* spp., using *Amaranthus hybridus* as test plant. *P. amaranthicola* infected *A. hybridus* at 20, 25, 30, and 35°C but the disease level achieved at 20°C may not be sufficient to cause high plant mortality. Plant mortality was also significantly lower in plants that were exposed to 4 h of dew. Plants at less than two- to two- to four-leaf stage were more easily killed than older plants, and increasing conidial concentration from 10^5 to 10^6 or 10^7 conidia ml^{-1} did not result in higher mortality levels. Among the adjuvants tested, polyalkyleneoxide-modified heptamethyltrisiloxane, algal polysaccharide, hydroxyethyl cellulose, and octylphenoxy polyethoxyethanol reduced conidial germination. Conidia applied with invert emulsion caused the highest plant mortality (74%) but invert emulsion alone caused 33% plant death due to phytotoxicity. Results indicate that *P. amaranthicola* can infect and kill *Amaranthus* spp. under a range of temperature, dew period, and inoculum levels and, therefore, has good potential as a bioherbicide agent.

Additional keywords: biological control, pigweed

The genus *Amaranthus* L. comprises numerous species of broadleaf plants that are commonly called pigweeds or amaranths. These species are predominantly herbaceous or sometimes woody annuals with a well-developed taproot that allows them to thrive under dry conditions. Although some species are considered to be excellent grain crops or leafy vegetables (7,22), some are regarded as agronomic pests in many countries of the world, causing significant reduction in the yield of crops such as sugar beet (*Beta vulgaris* (L.) Beauv.), sugar cane (*Saccharum officinarum* L.), potato (*Solanum tuberosum*

L.), wheat (*Triticum aestivum* L.), soybean (*Glycine max* (L.) Merr.), cotton (*Gossypium hirsutum* L.), coffee (*Coffea* spp.), oat (*Avena* spp.), peanut (*Arachis hypogaea* L.), sunflower (*Helianthus annuus* L.), upland rice (*Oryza sativa* L.), mango (*Mangifera indica* L.), sorghum (*Sorghum bicolor* (L.) Moench.), citrus (*Citrus* spp.), and papaya (*Carica papaya* L.) (14). *Amaranthus* spp. that are considered serious agronomic weeds include *Amaranthus hybridus* (smooth pigweed), *A. spinosus* (spiny amaranth), *A. retroflexus* (red-root pigweed), *A. viridis* (green amaranth), and *A. dubius* (spleen amaranth). In Florida, pigweeds are among the 10 most commonly found weeds in tobacco (*Nicotiana tabacum* L.), soybean, cotton, and peanut (8). Some *Amaranthus* spp. also can cause livestock poisoning due to the high levels of nitrate they contain (15).

Effective control of pigweeds or amaranths has been achieved with the use of triazine herbicides; however, resistant and cross-resistant weed populations have emerged through many years of herbicide use (4,12,13). Herbicide resistance, non-target effects, and toxicity problems are among the reasons that the use of biological agents has been considered as an option for pigweed management. *Phomopsis amaranthicola* Roskopf, Charudattan, Shabana & Benny is a promising biocontrol agent for pigweeds. This fungus was

isolated from blighted stems and leaves of an *Amaranthus* sp. collected from Gainesville, FL (26). Inoculations in the greenhouse produced leaf lesions that expanded and coalesced. Lesions that eventually reached the leaf petiole caused premature leaf abscission within 5 days of inoculation. Lesions also girdled the stem, causing stem constriction and toppling of plants. *P. amaranthicola* is pathogenic to *A. hybridus*, *A. spinosus*, *A. retroflexus*, *A. lividus*, and *A. viridis* (25).

Different pathogens require different temperature and moisture regimes for germination, infection, and sporulation. Although there is no broad generalization regarding the length of dew periods required by fungal biocontrol agents (31), the availability of moisture in the environment remains one of the most important factors for successful infection and colonization of the target weed by a biocontrol fungus. This study was conducted to determine how dew duration and dew-period temperature can influence the efficacy of *P. amaranthicola* against *A. hybridus*. The effects of other factors that can influence disease development and severity, such as plant growth stage or plant age, inoculum concentration, and addition of adjuvants, also were investigated.

MATERIALS AND METHODS

Propagation of test plants. *A. hybridus* (smooth pigweed) plants were used in this study because they were found to be moderately susceptible to infection by *P. amaranthicola* (unpublished data). Although other species, including *A. lividus* and *A. viridis*, were more susceptible, they were so severely affected by the pathogen that differences in the experimental parameters would not be detected. Smooth pigweed plants were grown from seed (Azlin Seed Service, Leland, MS) in the greenhouse and transplanted at the cotyledon stage to 9-cm-diameter clay pots (three plants per pot) containing Metromix 300 (Scott's-Sierra Horticultural Products Co., Marysville, OH). Plants were maintained in the greenhouse until they had two to four true leaves. The greenhouse used in this study had average day and night temperatures of 35 and 25 $\pm$ 5°C, respectively, an average relative humidity of 85 $\pm$ 5°C, and light intensity of 400 $\mu\text{mol m}^{-2} \text{s}^{-1}$ at midday.

Production and preparation of inoculum. *P. amaranthicola* was cultured on V8

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juice (Campbell Soup Co., Camden, NJ) agar in 100-by-15-mm petri plates. Plate cultures were incubated at $25 \pm 2^\circ\text{C}$ and with diurnal light. Conidial suspensions were prepared by flooding 12-to 24-day-old V8 juice agar cultures with 10 ml of deionized water and dislodging the conidia using a sterilized rubber policeman. The resulting suspension was filtered through two layers of sterile cheesecloth and standardized to the desired conidial concentrations using a hemacytometer. *P. amaranthicola* α conidia were used in all the experiments.

Effect of dew duration and dew-period temperature. A conidial suspension of *P. amaranthicola* was prepared and amended with 0.5% psyllium mucilloid (Metamucil; Procter and Gamble, Cincinnati, OH) and used to inoculate plants of the four- to six-leaf stage, using the method previously described. The experiment was done as a four-by-four factorial experiment; inoculated plants then were exposed to varying lengths of dew (4, 8, 12, and 24 h) inside dew chambers with temperatures of 20, 25, 30, and 35°C . Controls consisted of plants that were sprayed with 0.5% psyllium mucilloid suspension and then exposed to the same dew durations and temperatures as the inoculated plants. Each treatment consisted of three replicates, with three plants per replicate. Plant mortality data were taken as the proportion of dead plants at 8 weeks after inoculation. The experiment was conducted twice.

Effect of plant growth stage. The effect of plant growth stage on the development of disease and plant mortality was tested using plants of five growth stages: 1 = less than two-leaf stage, fully expanded cotyledon, with the first true leaves just beginning to open (approximately 10 days after planting); 2 = two- to four-leaf stage, two fully expanded leaves, with the third and fourth leaves just beginning to expand (approximately 16 days after planting); 3 = four- to six-leaf stage, four true leaves fully expanded and the fifth and six leaves beginning to expand (approximately 20 days after planting); 4 = six- to eight-leaf stage, six fully expanded leaves, with the seventh and eighth leaves just beginning to expand (approximately 26 days after planting); and 5 = flowering, axillary buds pre-

sent and producing flowers (approximately 30 days after planting). Plants were grown from seed in the greenhouse in a staggered fashion to accomplish the desired growth stages.

The experiment was done in a completely randomized design (CRD); each treatment consisted of three replicates, with three plants per replicate. Plants were sprayed with a handheld pump sprayer to incipient runoff with a conidial suspension containing 1×10^6 conidia ml^{-1} . Controls consisted of each plant growth stage treated with a 0.5% psyllium mucilloid suspension. Inoculated and control plants were incubated inside an unlit dew chamber for 24 h at $28 \pm 2^\circ\text{C}$ with 24 h of dew. Plant mortality data were taken as the proportion of dead plants at 8 weeks after inoculation. The experiment was conducted twice.

Effect of inoculum concentration. Conidial suspensions were diluted to contain approximately 1.5×10^5 , 6.0×10^5 , 1.5×10^6 , 6.0×10^6 , and 1.5×10^7 conidia ml^{-1} , as determined with a hemacytometer. All suspensions were amended with 0.5% psyllium mucilloid. Test plants at the four- to six-leaf stage were inoculated with a handheld pump sprayer until incipient run-off and were then exposed to 12 h of dew at $25 \pm 2^\circ\text{C}$ in the dark. Inoculated and control plants (0 conidia ml^{-1}) were moved to the greenhouse and observed for disease development and plant mortality. The experiment was done in a CRD; each treatment consisted of three replicates, with three plants per replicate. Plant mortality data were taken as the proportion of dead plants at 8 weeks after inoculation. The experiment was conducted twice.

Effect of adjuvants on biocontrol efficacy. Conidial suspensions of *P. amaranthicola* were mixed with eight different types of adjuvants (Table 1). The invert emulsion (IE) was prepared according to Yang et al. (38); it was composed of 39.2% horticultural oil, 9.8% light mineral oil, 0.98% emulsifying agent, and 50% water. A laboratory test was conducted to determine whether the adjuvants reduced the number of viable *P. amaranthicola* α conidia. Conidia suspended in water only and conidial suspensions containing each of the adjuvants were sprayed onto water agar

plates with 2% sucrose using a compressed air sprayer (138 kPa). The inoculated plates were incubated in ambient light at 25°C for 24 h and then checked for germinating conidia. The experiment was done in a CRD, with three replications per treatment. The proportion of germinating conidia was determined from 50 conidia from each of the three replicate plates for each amendment. This experiment was performed three times.

The effect of the adjuvants on the efficacy of *P. amaranthicola* on smooth pigweed was determined by spraying the amended conidial suspensions onto plants at the four-leaf stage. Inoculation was performed using hand-pumped sprayers. Control plants were treated with each of the adjuvants mixed with water only. All test plants were exposed to 12 h of dew at $25 \pm 2^\circ\text{C}$ and then transferred to the greenhouse for observation. The experiment was done in a CRD; each treatment consisted of three replicates, with three plants per replicate. Plant mortality data were taken as the proportion of dead plants at 8 weeks after inoculation. The experiment was conducted three times.

Data analysis. Data from similar trials were pooled together when the variances were found to be homogenous; otherwise, data from similar trials were analyzed separately. The proportions were transformed using the arcsine square-root transformation. Analysis of variance (ANOVA) was performed using the General Linear Model Procedure of SAS (SAS Institute, Inc., Cary, NC) to determine the effects of the factors tested. When appropriate, means were separated using Duncan's Multiple Range test ($P = 0.05$).

RESULTS

Effect of dew duration and dew-period temperature. Data from two trials were analyzed separately due to unequal variances. However, results from the separate analysis were similar. Temperature and dew period length had significant effects on plant mortality but there was no interaction effect between these two factors. Plant mortality was significantly lower when the incubation temperature was 20°C and when the dew period was 4 h (Figs. 1 and 2). The temperature conducive for high plant mortality was between 25

Table 1. Brand name, concentration, composition, and manufacturer of adjuvants added to *Phomopsis amaranthicola* conidia

Adjuvant	Concentration and composition	Manufacturer
Invert emulsion	39.2%; Sunspray 6 horticultural oil 9.8%; Light mineral oil 0.98%; Myverol (monoglyceride emulsifying agent)	Sun Co., Inc., Marcus Hook, PA Fisher Scientific, Fair Lawn, NJ Eastman Chemical Co., Kingsport, TN
Kelgin	0.05%; Algal polysaccharide composed of sodium polymannuronate	Nutrasweet Kelco Co., San Diego, CA
Kelzan	0.05%; Xanthan gum	Nutrasweet Kelco Co., San Diego, CA
Metamucil	0.05%; Psyllium mucilloid	Procter and Gamble, Cincinnati, OH
Natrasol	0.05%; Hydroxyethyl cellulose	Aqualon Co., Wilmington, DE
Silwet L-77	0.25%; Polyalkyleneoxide modified heptamethyltrisiloxane	Loveland Industries, Inc., Greeley, CO
Triton X-100	0.05%; Octylphenoxy polyethoxyethanol, non-ionic surfactant	Rohm and Haas, Philadelphia, PA
Tween 20	0.3%; Polyoxyethylene sorbitan monolaurate	Sigma-Aldrich, St. Louis, MO

and 35°C, whereas the optimum dew period was between 8 and 12 h.

Effect of plant growth stage. The growth stage of *A. hybridus* significantly influenced plant mortality levels ($P < 0.0001$). Mortality was decreased in older plants (from four- to six-true-leaf stage to flowering stage; stages 3 to 5) than in younger plants (less than two-true-leaf and two- to four-true-leaf stages; stages 1 and 2) (Fig. 3).

Effect of conidial concentration. The different concentrations of conidia of *P. amaranthicola* (1.5×10^5 , 6.0×10^5 , 1.5×10^6 , 6.0×10^6 , and 1.5×10^7 conidia ml^{-1}) applied to *A. hybridus* caused plant mortality levels that were not significantly different from one another ($P = 0.05$). The proportion of dead plants ranged from 0.26 to 0.37 (data not shown). The only difference observed was between the control treatment, consisting of the application of psyllium mucilloid alone, and the treatments containing conidia.

Effect of adjuvants on biocontrol efficacy. Psyllium mucilloid (Metamucil; META), polyoxyethylene sorbitan monolaurate (Tween 20), IE, and xanthan gum (Kelzan) did not inhibit or enhance the germination of *P. amaranthicola* compared with water control (Fig. 4). However, polyalkyleneoxide-modified heptamethyltrisiloxane surfactant (Silwet L-77) inhibited germination of α conidia, resulting in very low germination. The germination of α conidia mixed with algal polysaccharide (Kelgin), hydroxyethyl cellulose (Natralsol), and octylphenoxy polyethoxyethanol nonionic surfactant (Triton X-100) also was significantly lower than the germination of conidia suspended in water alone.

The type of adjuvant applied with *P. amaranthicola* conidia influenced *A. hybridus* mortality levels ($P < 0.0001$; Fig. 5). Plants sprayed with *P. amaranthicola* conidia suspended in IE had the greatest proportion of dead plants (0.74). Among all the additives tested, IE alone (without conidia) caused phytotoxic damage which resulted in plant mortality (0.33); however, this was significantly lower than the plant mortality caused by the IE with conidia (Fig. 5). None of the other additives enhanced *P. amaranthicola* efficacy and had no detrimental effects on the test plants when applied alone.

DISCUSSION

This study demonstrated that *P. amaranthicola* can infect and cause disease at any of the temperatures tested (20, 25, 30, and 35°C) and that disease severity may or may not increase dramatically when inoculated plants are exposed to dew periods greater than 8 h. The temperature range at which *P. amaranthicola* caused disease on smooth pigweed in this study is similar to the optimal temperature range reported for several other *Phomopsis* spp. (11,27,32). The most important implication of these

results is that the temperature range at which *P. amaranthicola* can infect smooth pigweed falls within the temperature range during the growing season of the weed in the southeastern United States. However, although disease did occur at 20°C, it seems that the disease is not severe enough to cause mortality or high levels of mortality. The apparent reduction in the efficacy of *P. amaranthicola* in cooler temperature suggests that it may be suitable for controlling pigweeds in northern crops, such as beet, only during the summer months (28,29). However, it is unlikely that the use

of this fungus in more southern temperate and subtropical regions will be limited by temperature.

This study also indicates that a minimum dew period of 8 h may be necessary to cause an appreciable level of control. Although this 8-h period is relatively short compared with the dew period requirement of many other potential biological control agents (20,23,37,38), it is still a longer period than what is normally available in the field. Hence, it is important to time the application of *P. amaranthicola* with periods when there is sufficient moisture to

Fig. 1. Mortality of inoculated plants incubated at 20, 25, 30, and 35°C with increasing duration of dew exposure (4, 8, 12, and 24 h), first trial.

Fig. 2. Mortality of inoculated plants incubated at 20, 25, 30, and 35°C with increasing duration of dew exposure (4, 8, 12, and 24 h), second trial.

Fig. 3. Effect of plant growth stage on the efficacy of *Phomopsis amaranthicola* as a biocontrol agent for *Amaranthus hybridus*. Graph represents data from two trials. Plant growth stage was based on the number of fully expanded leaves or the initiation of flowering. Bars with the same letters are not significantly different from each other at $P = 0.05$, as determined by Duncan's Multiple Range test.

Fig. 4. Effect of various adjuvants on the viability of *Phomopsis amaranthicola* conidia. The adjuvants tested were IE = invert emulsion, META = Metamucil, KEL = Kelzan, WAT = water, SIL = Silwet L-77, TWN = Tween 20, TRI = Triton X-100, NAT = Natrasol, and KELG = Kelgin. Viability was measured in terms of proportion of germinated conidia. Graph shows data from three trials. Bars with the same letters are not significantly different from each other at $P = 0.05$, as determined by Duncan's Multiple Range test.

ensure infection and disease development on pigweeds. In field trials of *P. amaranthicola* against smooth pigweed and other *Amaranthus* spp., Roskopf et al. (25,26) obtained high levels of infection, plant mortality, and secondary disease spread within and between plots. These results confirmed the ability of *P. amaranthicola*

to infect and incite disease and an epidemic under natural field conditions. Therefore, although our present results from the controlled study indicated a dew-duration requirement of >8 h, the fungus may be able to infect and spread under repeated cycles of <8 h of dew that is obtainable under field conditions.

The growth stage of the *A. hybridus* seedlings appeared to be one of the more significant factors affecting the efficacy of *P. amaranthicola*. The importance of early-season control of *Amaranthus* spp. has been documented for several crops, including bean (17) and soybean (18). The ability of *P. amaranthicola* to control pigweeds early in their growth is compatible with the need for early-season control of these weeds in many crops (16,33,34). Although the most susceptible growth stages are the smaller stages, it may be possible to improve the efficacy of *P. amaranthicola* on older or more mature plants with the addition of certain adjuvants that can enhance biocontrol efficacy.

In this study, relatively low concentrations of conidia (10^5 conidia mL^{-1}) were found to be adequate for causing mortality of *A. hybridus* plants. Unlike other potential agents for biological control of weeds, such as *P. convolvulus*, which requires 10^9 conidia/ml to cause high levels of mortality in seedlings of field bindweed (19), there were no significant differences between applications of different concentrations of conidia (10^5 , 10^6 , or 10^7 conidia mL^{-1}).

The timing of application of *P. amaranthicola* as a biological control agent for pigweeds is decidedly one of the most important factors for consideration. Timing plays a crucial role, both in terms of the growth of the target weeds and in optimization of the environmental conditions that are present during and after application. It appears that applications made early after weed emergence would be most conducive for effective control. The pathogen also should be applied to take advantage of the most humid periods or timed to coincide with the onset of the evening or early-morning dew period. These factors are more important to consider than the temperature at the time of application.

The various additives tested in this study reduced, inhibited, or did not have any effect on the germination of *P. amaranthicola* conidia. Although the IE did not seem to enhance germination of α conidia, the highest plant mortality was achieved when the conidia were applied with the IE. An IE is composed of water surrounded by a layer of oil; the oil improves the adhesion of the conidia to the host surface while the water provides the moisture needed by the conidia for germination (9,10,21,35,36). Increased efficacy through the addition of IE also has been reported for other biocontrol agents, such as *Alternaria cassiae* (24) and *Colletotrichum truncatum* (6). However, IEs generally are toxic to plants (3,5,35), highly viscous and incompatible with spray equipment, and are not easy to prepare. Because of these reasons, attention recently has been focused on other materials that can enhance efficacy but are less phytotoxic and easier to prepare and apply.

Fig. 5. Effect of various adjuvants on the bioherbicidal efficacy of *Phomopsis amaranthicola*. The adjuvants tested were IE = invert emulsion, META = Metamucil, KEL = Kelzan, WAT = water, SIL = Silwet L-77, TWN = Tween 20, TRI = Triton X-100, NAT = Natrasol, and KELG = Kelgin. Efficacy was measured in terms of proportion of dead *Amaranthus hybridus* plants. Graph shows data from three trials (gray bars show the effect of conidia + adjuvant and black bars show the effect of the adjuvant alone). Bars with the same letters are not significantly different from each other at $P = 0.05$, as determined by Duncan's Multiple Range test.

Variable efficacy of *P. amaranthicola* was observed between trials and between the experiments in this study. This indicates that, aside from dew duration, dew-period temperature, and plant age, there are other factors that influenced the ability of *P. amaranthicola* conidia to germinate, cause disease, or kill *Amaranthus hybridus*. There is evidence that cultural conditions (i.e., type of culture media or temperature during incubation of cultures) can affect a pathogen's ability to cause disease (1,2) and that the conidial matrix produced by some species of *Phomopsis* can inhibit the germination of conidia (30); however, the determination of how much these factors can affect biocontrol efficacy of *P. amaranthicola* was beyond the scope of this study.

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